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Differential retrotranslocation of mitochondrial Bax and Bak

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Abstract

The Bcl-2 proteins Bax and Bak can permeabilize the outer mitochondrial membrane and commit cells to apoptosis. Pro-survival Bcl-2 proteins control Bax by constant retrotranslocation into the cytosol of healthy cells. The stabilization of cytosolic Bax raises the question whether the functionally redundant but largely mitochondrial Bak shares this level of regulation. Here we report that Bak is retrotranslocated from the mitochondria by pro-survival Bcl-2 proteins. Bak is present in the cytosol of human cells and tissues, but low shuttling rates cause predominant mitochondrial Bak localization. Interchanging the membrane anchors of Bax and Bak reverses their subcellular localization compared to the wild-type proteins. Strikingly, the reduction of Bax shuttling to the level of Bak retrotranslocation results in full Bax toxicity even in absence of apoptosis induction. Thus, fast Bax retrotranslocation is required to protect cells from commitment to programmed death.

Keywords apoptosis; Bcl-2 proteins; membrane association; tail anchor
Subject Categories Autophagy & Cell Death
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Introduction

In response to stress, cells can initiate mitochondrial apoptosis signaling in multicellular animals. The intrinsic cell suicide program converges at the activation of the Bcl-2 proteins Bax and Bak that permeabilize the outer mitochondrial membrane (OMM). The release of cytochrome *c* (cyt *c*) and other intermembrane space proteins into the cytosol after OMM permeabilization (mitochondrial outer membrane permeabilization, MOMP) results in mitochondrial dysfunction and initiates the caspase cascade that efficiently dismantles the cell (Bratton & Cohen, 2001; Green & Kroemer, 2004). The activation of Bax or Bak commits the cell to apoptosis necessitating a tight control of pro-apoptotic Bcl-2 proteins (Lindsten *et al*, 2000). Thus, the Bcl-2 protein family contains two groups of structurally similar proteins involved in arbitration to apoptosis: pro-survival Bcl-2 proteins harboring four Bcl-2 homology domains (BH1-4, e.g. Bcl-2, Bcl-x_L or Mcl-1) and pro-apoptotic Bcl-2 proteins with three BH domains (BH1-3, e.g. Bax or Bak). Proteins of both groups are regulated by a diverse group of proteins sharing only the BH3 domain with Bcl-2 proteins (BH3-only proteins). Pro-survival Bcl-2 proteins inhibit Bax and Bak via direct interactions or by sequestering 'activator' BH3-only proteins, thereby preventing their interaction with Bax and Bak (Letai *et al*, 2002; Kuwana *et al*, 2005; Willis *et al*, 2005, 2007; Kim *et al*, 2006; Llambi *et al*, 2011). Regulatory interactions between pro- and anti-apoptotic Bcl-2 proteins and Bax and Bak can only be observed in the presence of the OMM or liposomes and could result in membrane-integral protein complexes (Roucou *et al*, 2002), suggesting also mitochondrial apoptosis signaling via membrane-embedded proteins (Leber *et al*, 2007; Lovell *et al*, 2008; García-Sáez *et al*, 2009). Upon apoptosis induction, Bax and Bak oligomerize and at least partially insert into the OMM (Eskes *et al*, 1998; Antonsson *et al*, 2000; Wei *et al*, 2001).

The C-terminal Bax and Bak segments are sufficient for targeting protein fusions to the OMM, whereas deletion of the C-terminal segments abolishes mitochondrial localization and pro-apoptotic activity of Bax and Bak (Nechushtan *et al*, 1999; Schinzel *et al*, 2004; Setoguchi *et al*, 2006). C-terminal membrane anchors (MAs) direct proteins post-translationally to the target organelle and may insert into the membrane (Habib *et al*, 2003). Single amino acid substitutions in the C-terminal helix can either localize Bax almost

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completely to the mitochondria or prevent OMM binding at all (Nechushtan *et al*, 1999). The potential binding site of Bax for pro-apoptotic BH3 motifs interacts with its MA in cytosolic Bax inhibiting MA exposure and binding to BH3 motifs (Suzuki *et al*, 2000). The exposure of the C-terminal Bax helix may involve prolyl isomerization in the loop preceding the MA or BH3-only protein binding to a low affinity site in the N-terminal part of Bax (Schinzel *et al*, 2004; Gavathiotis *et al*, 2008).

Bak could be controlled by mechanisms similar to Bax regulation, considering the dependence of activation and oligomerization of both proteins on major conformational changes, exposing the BH3 motifs (Wang *et al*, 1998; Dewson *et al*, 2008; Edlich *et al*, 2011; Moldoveanu *et al*, 2013). The conversion of inactive into active Bak seems to depend exclusively on transient BH3-only protein interactions with the hydrophobic cleft of Bak (Dai *et al*, 2011). In healthy cells, Bax primarily resides in the cytosol (Wolter *et al*, 1997), contrasting with the predominant mitochondrial Bak localization. Although Bax is constantly translocating to the OMM, it is stabilized in the cytosol by interactions with pro-survival Bcl-2 activities on the OMM, establishing an equilibrium between cytosolic and mitochondrial Bax (Edlich *et al*, 2011; Schellenberg *et al*, 2013). Bax retrotranslocation from the mitochondria requires recognition of its exposed BH3 motif by the hydrophobic groove of Bcl-x_L and interaction between the C-terminal Bcl-x_L helix and Bax (Edlich *et al*, 2011; Todt *et al*, 2013). Bax shuttling could also involve Bcl-x_L-independent mechanisms (Schellenberg *et al*, 2013). When Bax retrotranslocation is compromised, Bax accumulates on the OMM, but requires further stimulation to become active dependent on the size of the mitochondrial Bax pool prior to apoptosis stimulation (Todt *et al*, 2013). Reversible mitochondrial Bax accumulation can be observed during anoikis (Valentijn *et al*, 2003).

The functional redundancy between Bax and Bak raises the question, why Bak is predominantly found on the mitochondria. Are Bax and Bak controlled by different mechanisms or does differential regulation of the same mechanism cause different localizations of these pro-apoptotic Bcl-2 proteins? Considering that resistance to apoptosis is one hallmark of cancer (Hanahan & Weinberg, 2011), understanding the regulation of Bax and Bak is imperative for targeting the mitochondrial apoptosis pathway in cancer therapy.

Results

Bak is present in the cytosol

The importance of the subcellular localization for the regulation of Bax raises the question whether Bak is also regulated by shuttling between mitochondria and cytosol in spite of its predominant mitochondrial localization. Thus, the presence of Bak in the cytosol (C) and the mitochondria-containing heavy membrane fraction (HM) from human tissues was analyzed with specific antibodies (Fig 1A and B). Different levels of Bak are present on the mitochondria of all tissue samples (Fig 1C). Strikingly, in heart, kidney and lung tissue, Bak is also present in the cytosolic fraction (Fig 1D; Supplementary Fig S1). Interestingly, human lung tissue not only shows cytosolic Bak but the HM fraction also contains membrane-associated Bak in addition to membrane-integral protein (Fig 1E). The presence of Bak in the cytosol of human tissues raises the question, whether Bak is shuttled into the cytosol by a mechanism similar to Bax retrotranslocation (Edlich *et al*, 2011; Todt *et al*, 2013).

Bcl-x_L retrotranslocates Bak

We tested the possibility of Bak shuttling by Fluorescence Loss in Photobleaching (FLIP) measurements of mitochondrial Bak with and without ectopically expressed Bcl-x_L. The experiments were performed with transient protein expression in HCT116 Bax/Bak DKO cells to avoid high protein levels resulting from stable protein expression (Supplementary Fig S2A–D). FLIP experiments target cytosolic fluorescence by repeated cycles of bleaching within a defined region, while changes in mitochondrial fluorescence are monitored by confocal imaging between bleaching events (Ishikawa-Ankerhold *et al*, 2012).

GFP-Bak localizes to the mitochondria but cytosolic Bak is apparent when Bcl-x_L is overexpressed (Fig 2A). After the cytosolic fluorescence was bleached within the initial cycles, mitochondrial GFP-Bak fluorescence diminished during the FLIP measurement indicating Bak shuttling (Fig 2B and C). Retrotranslocation of mitochondrial GFP-Bak is increased by Bcl-x_L, accelerating the loss of mitochondrial fluorescence during the measurements (Fig 2B and C, arrows). Therefore, Bcl-x_L retrotranslocates mitochondrial Bak into the cytosol. Low Bak shuttling rates could explain the differential localization of Bax and Bak in cells (Table 1).

The same retrotranslocation process shuttles Bax and Bak

Bcl-x_L increases Bak shuttling. Therefore, the effect of wild-type Bcl-x_L or Bcl-x_L G138A on Bak localization was analyzed in HCT116 Bax/Bak DKO cells. The G138A substitution prevents Bax BH3 binding to the hydrophobic groove of Bcl-x_L and Bax retrotranslocation (Sedlak *et al*, 1995; Desagher *et al*, 1999; Edlich *et al*, 2011). While Bak is robustly found in the HM fraction, an additional Bak pool is also present in the cytosol (Fig 3A and B; Supplementary Fig S3A), corroborating the Bak localization in human tissues (Fig 1). Elevated levels of wild-type Bcl-x_L, but not Bcl-x_L G138A, increase the cytosolic Bak pool (Fig 3A and C; Supplementary Fig S3A and B). Increased levels of Mcl-1 accelerate Bak retrotranslocation similar to Bax shuttling (Fig 3C; Edlich *et al*, 2011). On the other hand, the BH3 mimetic ABT-737 decreases Bak retrotranslocation in the presence of ectopically expressed Bcl-x_L (Supplementary Fig S3C). In contrast to previously observed Bax shuttling (Edlich *et al*, 2011), ectopic Bcl-2 expression does not accelerate Bak retrotranslocation.

The D83R substitution prevents Bak BH3 interactions with pro-survival Bcl-2 proteins (Kvansakul *et al*, 2007). Consistent with the effects of ABT-737 and Bcl-x_L G138A, the Bak D83R substitution inhibits pro-survival Bcl-2 protein-mediated Bak shuttling (Fig 3C). Therefore, interactions between the BH3 motif and hydrophobic groove of Bcl-x_L are required for Bak/Bax retrotranslocation. In parallel to ectopically expressed Bak, endogenous Bak is significantly increased in the cytosol of HeLa cells when Bcl-x_L levels are elevated (Fig 3D; Supplementary Fig S3D and E). Cytosolic Bak results from constant shuttling and is not diminished when protein synthesis is blocked (Supplementary Fig S3F). Thus, different Bak shuttling rates can account for the differential localization of Bak in human tissues (Fig 1).

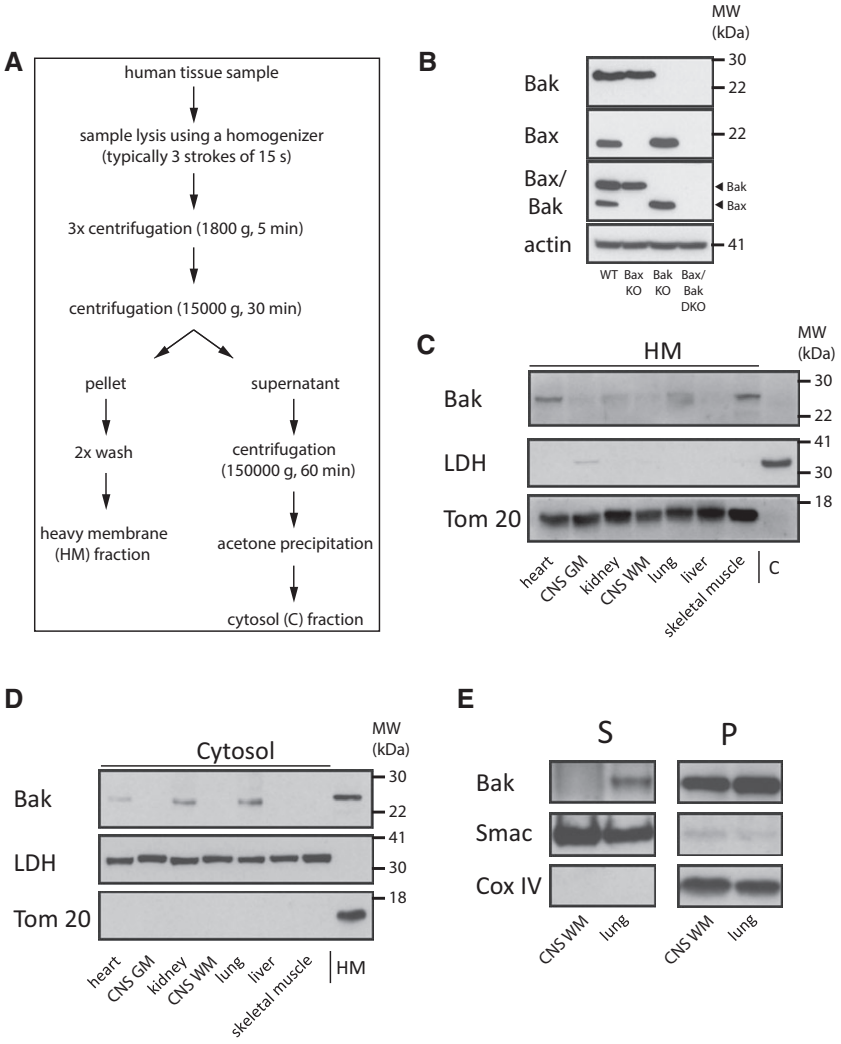

Figure 1. Bak is present in the cytosol.

A Flow chart of human tissue homogenization and subsequent fractionation into cytosolic (C) and mitochondria-containing heavy membrane (HM) fraction. With authorization by the local ethics committee, human tissue samples were obtained from the Institute of Pathology, University Hospitals of Basel.

B Analysis of anti-Bax and anti-Bak antibody specificity using whole-cell lysates from HCT116 wild-type, Bax KO, Bak KO and Bax/Bak DKO. After detection of Bax the membrane was redecorated with anti-Bak antibodies (Bax/Bak). Actin is used as a loading control.

C Western blot analysis of Bak in the heavy membrane (HM) fraction of human heart, cerebral cortex (CNS GM), kidney, brain white matter (CNS WM), lung, liver, and skeletal muscle tissue. The cytosol fraction obtained from CNS cortex (C), LDH, and Tom20 serve as fractionation and loading controls. $n = 3$.

D Presence of Bak in the cytosol of heart, cerebral cortex (CNS GM), kidney, brain white matter (CNS WM), lung, liver, and skeletal muscle tissue analyzed by Western blot in parallel to (C). The heavy membrane heart fraction (HM), LDH and Tom20 serve as fractionation and loading controls. $n = 3$.

E Carbonate extraction of the heavy membrane protein fraction of human brain white matter (CNS WM) and lung tissue analyzed for the presence of Bak by Western blot. Membrane-associated protein supernatant (S) and membrane-integral protein pellet (P) are displayed. Smac is released from the mitochondrial intermembrane space (IMS) during carbonate extraction and CoxIV remains in the pellet. $n = 2$.

Source data are available online for this figure.

Bax and Bak shuttling is determined by the membrane anchor

Single amino acid substitutions in the C-terminal MA of Bax can shift the protein to the mitochondria and decelerate its retrotranslocation (Nechushtan *et al*, 1999; Edlich *et al*, 2011; Schellenberg *et al*, 2013). Therefore, the impact of MA substitutions on the subcellular localization and retrotranslocation of Bax and Bak was analyzed (Fig 4A). Both chimeras (BaxTBak and BakTBax) were expressed to levels comparable to the wild-type proteins in the presence or the absence of ectopically expressed Bcl-x_L (Supplementary Fig S4A and B). While Bax resides in most cells primarily in the cytosol, BaxTBak is largely localized to the mitochondria, similar to the localization of wild-type Bak (Fig 4B and C). Interestingly, HCT116 Bax/Bak DKO cells expressing BaxTBak often show a punctate fluorescence pattern (Fig 4C), as is characteristic for active Bax (Karbowski *et al*, 2002). BakTBax, on the other hand, exhibits a large pool of cells with significant amounts of cytosolic protein, corroborating the central role of the Bak MA in protein localization

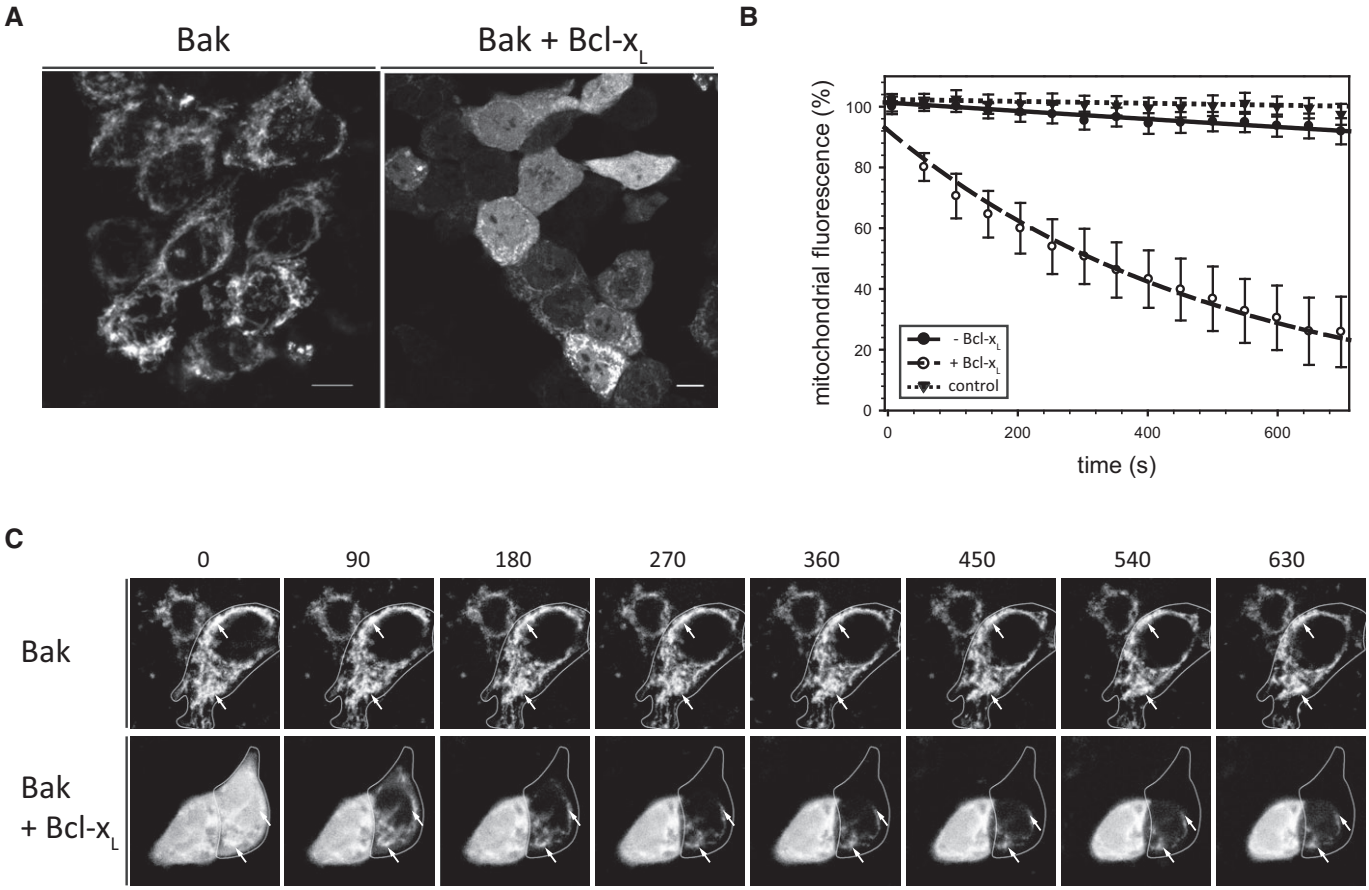

Figure 2. Bcl-x_L retrotranslocates Bak.

A Confocal images of HCT116 Bax/Bak DKO cells transfected with GFP-Bak in the absence (left) or the presence (right) of Bcl-x_L overexpression. Scale bar, 15 μm. $n \geq 10$.

B FLIP (Fluorescence Loss in Photobleaching) of mitochondrial GFP-Bak in the absence (●, straight line) or the presence (○, broken line) of overexpressed Bcl-x_L. Fluorescence of the neighboring cell (C) is shown as a control (▼, dotted line). Data represent averages ± SEM from 16 (-Bcl-x_L) and 40 (+Bcl-x_L) ROI measurements.

C FLIP of GFP-Bak in the absence (top) or the presence (bottom) of overexpressed Bcl-x_L diminishes GFP-Bak fluorescence in the cytosol of targeted cells (circled) completely after 90 s and GFP fluorescence is detected only on the mitochondria (arrows). During FLIP measurements mitochondrial GFP-Bak fluorescence is monitored, while the cytosol is bleached repeatedly. Time points in seconds are displayed above the images.

Table 1. Retrotranslocation rates of Bax/Bak variants.

	K_{obs} (10^{-3} s^{-1})-Bcl-x_L	K_{obs} (10^{-3} s^{-1}) + Bcl-x_L
Bax	4.68 ± 0.11 (23)	8.61 ± 0.37 (23)
Bax S184V	3.48 ± 0.20 (23)	5.90 ± 0.13 (23)
BaxTBak	0.43 ± 0.10	2.31 ± 0.67
BaxTBakSS	3.91 ± 0.25	6.32 ± 0.65
Bak	0.14 ± 0.02	1.94 ± 0.09
BakTBax	3.68 ± 0.06	5.71 ± 0.19
BakTBax S184V	3.25 ± 0.22	5.30 ± 0.37

Retrotranslocation rates determined by FLIP measurements of different Bax/Bak variants ± SD as depicted in Figs 2B and 6A, Supplementary Figs S3C and D and S6B compared to rates of wild-type Bax and Bax S184V (Edlich *et al*, 2011).

(Ferrer *et al*, 2012). However, the different localization observed for BakTBax chimeras (Fig 4C and Ferrer *et al*, 2012) could be caused by differences in MA composition, protein expression and the resulting cell stress. Despite increased mitochondrial BakTBax levels compared to wild-type Bax, cell fractionations substantiated greater similarity between BakTBax and wild-type Bax localization compared to Bak (Fig 4B–D).

In parallel to localization, the shuttling of BaxTBak and BakTBax is altered compared to their wild-type counterparts. Bax retrotranslocation is slowed down to the level of Bak shuttling by the Bak MA, while Bak shuttling is significantly accelerated by the Bax MA (Supplementary Fig S4C and D; Table 1). These measurements show that the C-terminal membrane anchor determines retrotranslocation rates, and thus, localization of Bax and Bak.

Increased retrotranslocation is required to protect cells from Bax activation

The size of the mitochondrial Bax pool determines cellular commitment to apoptosis (Todt *et al*, 2013). The pronounced influence of shuttling rates on the subcellular localization of Bax and Bak suggests that both proteins are regulated by the rate of

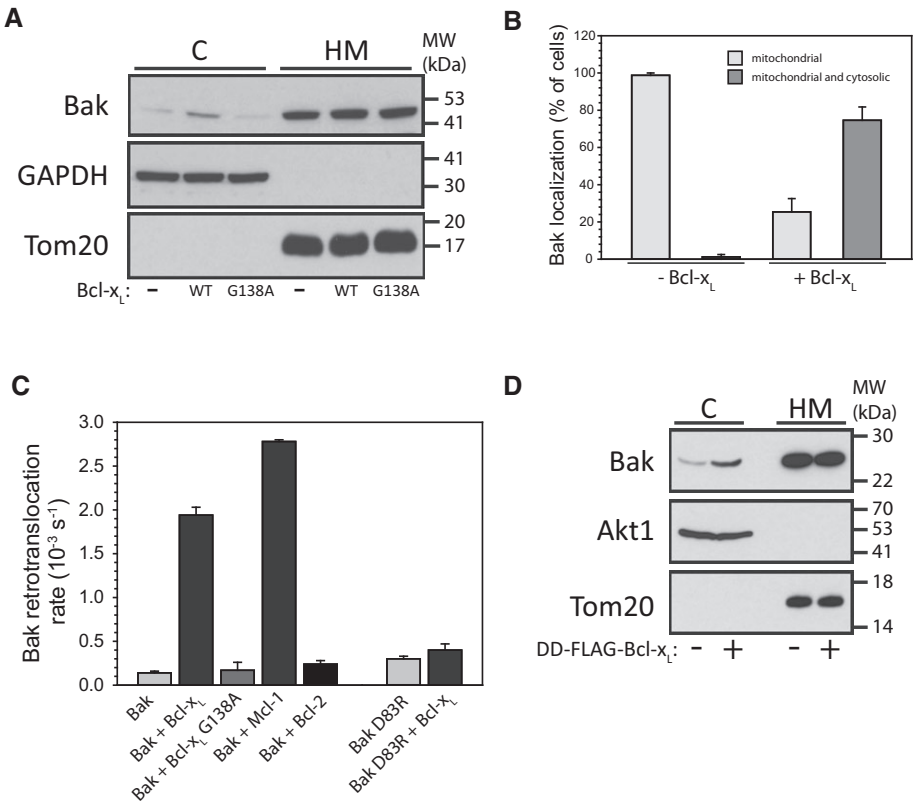

Figure 3. Bak is shifted into the cytosol in parallel to Bax by retrotranslocation.

A Western blot analysis of GFP-Bak localization in cytosol (C) and heavy membrane fraction (HM) of HCT116 Bax/Bak DKO cells with or without overexpressed wild-type Bcl-x_L or Bcl-x_L G138A. GAPDH and Tom20 serve as fractionation controls. $n = 10$. Bak shuttling by Bcl-x_L is independent of the GFP fusion (Supplementary Fig S2A).

B Quantification of predominant mitochondrial localization (light grey bars) and mixed cytosolic and mitochondrial localization (dark grey bars) of Bak in HCT116 Bax/Bak DKO cells expressing GFP-Bak with or without Bcl-x_L overexpression. Data represent averages ± SD from three independent experiments with $n \geq 100$.

C Retrotranslocation rates measured for wild-type Bak or Bak D83R in the presence or the absence of Bcl-2, Mcl-1, wild-type Bcl-x_L or Bcl-x_L G138A. Data represent averages ± SD.

D Analysis of endogenous Bak localization in cytosol (C) and heavy membrane fraction (HM) of HeLa cells expressing degradation-prone DD-FLAG-Bcl-x_L. In the absence of Shield-1 DD-FLAG-Bcl-x_L is readily degraded, resulting in unaltered Bcl-x_L levels. Addition of 0.5 μM Shield-1 instantly stabilizes DD-FLAG-Bcl-x_L resulting in elevated Bcl-x_L levels that increase cytosolic levels of endogenous Bak (Supplementary Fig S3C and D). Akt1 and Tom20 serve as fractionation controls. $n = 3$.

Source data are available online for this figure.

mitochondrial-cytosolic shuttling. Thus, the apoptotic activities of Bax, BaxTBak, Bak, or BakTBax ectopically expressed in HCT116 Bax/Bak DKO cells were analyzed by measuring caspase-3/7 activity and the cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP). In the absence of staurosporine (STS), Bax, Bak and BakTBax expression results in low caspase activity that can be inhibited by Bcl-x_L overexpression (Fig 5A; Supplementary Fig S5A). Strikingly, BaxTBak gains full activity in the absence of apoptotic stimuli that is not completely inhibited by Bcl-x_L overexpression, contrasting the regulation of both wild-type proteins.

In response to STS-induced apoptosis, BaxTBak activity in the presence of Bcl-x_L overexpression is even higher than Bax, Bak, or BakTBax activities in the absence of overexpressed Bcl-x_L (Fig 5B; Supplementary Fig S5B). Bcl-x_L fails to prevent the transition towards the active conformation of BaxTBak and cytochrome *c* release despite robust binding to the Bax variant (Fig 5C and D; Supplementary Fig S5C). Both wild-type proteins and BakTBax can be inhibited to basal apoptotic activity by Bcl-x_L overexpression. The Bak activity is reduced when containing the Bax MA. These results establish the link between increased retrotranslocation rates and reduced pro-apoptotic activity of Bax and Bak but show also the requirement for accelerated Bax shuttling.

Ectopic Bcl-x_L expression reduces the pool of Annexin V-positive cells expressing Bax, Bak or BakTBax but not that of cells expressing BaxTBak (Fig 5E). Colony formation after apoptosis stimulation in the presence of Bax, BaxTBak, Bak or BakTBax reflects the results of caspase-3/7 activity measurements and PARP cleavage (Fig 5F and G). In the presence of BaxTBak the predominant fate is cell death even when Bcl-x_L was overexpressed. Bcl-x_L also fails to protect cells from BaxTBak activity in the absence of apoptotic stimuli (Fig 5H). Thus, slow Bax retrotranslocation commits cells to apoptosis.

Bax and Bak are shuttled depending on the MA hydrophobicity

Apoptosis activity measurements revealed a striking difference between BaxTBak and Bak despite similar localization and shuttling

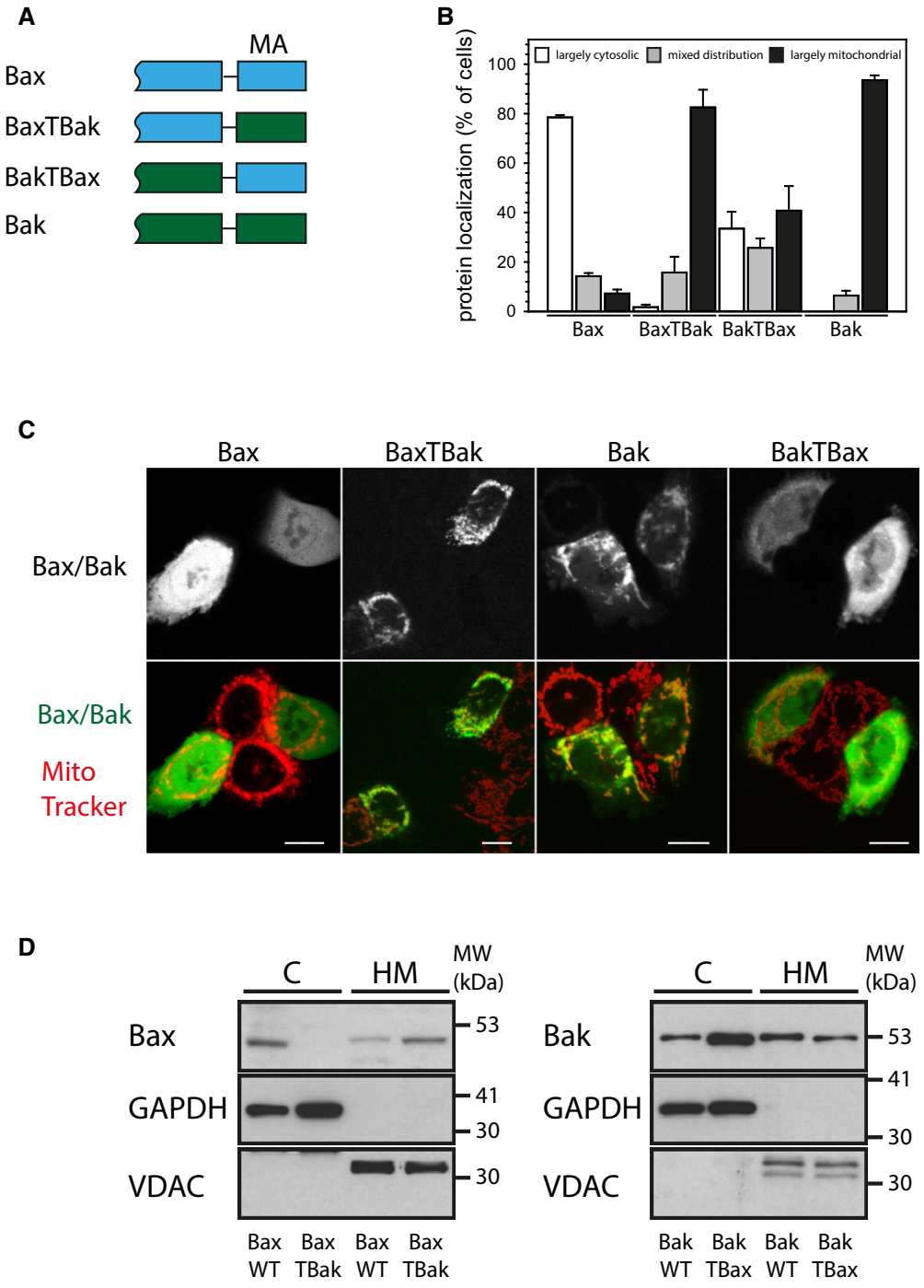

Figure 4. The C-terminal membrane anchor determines the localization of Bax and Bak.

A The influence of the C-terminal membrane anchor (MA) on the differential localization and function of Bax and Bak has been analyzed using the wild-type proteins of Bax (blue) and Bak (green) and MA substitutions of both proteins resulting in the chimeras BaxTBak and BakTBax.

B Quantification of HCT116 Bax/Bak DKO cells expressing Bax, BaxTBak, BakTBax or Bak with the expressed protein being present largely cytosolic (white bars), in a mixed distribution between cytosol and mitochondria (grey bars) or largely mitochondrial (black bars). Data represent averages ± SEM from seven independent experiments with $n \geq 100$ cells.

C Confocal images of HCT116 Bax/Bak DKO cells expressing Bax, BaxTBak, BakTBax or Bak. The GFP/YFP fluorescence of the expressed protein variants is depicted in the top panels and in green in the merged image on the bottom. The mitochondria were stained by MitoTracker far red depicted in red in the merged images (bottom row). Scale bar, 10 μm. $n \geq 5$.

D Western blot analysis of Bax, BaxTBak, Bak and BakTBax localization expressed in HCT116 Bax/Bak DKO cells. Cytosol (C) and heavy membrane fraction (HM) of HCT116 Bax/Bak DKO cells are displayed. GAPDH and VDAC serve as fractionation controls. $n = 3$.

Source data are available online for this figure.

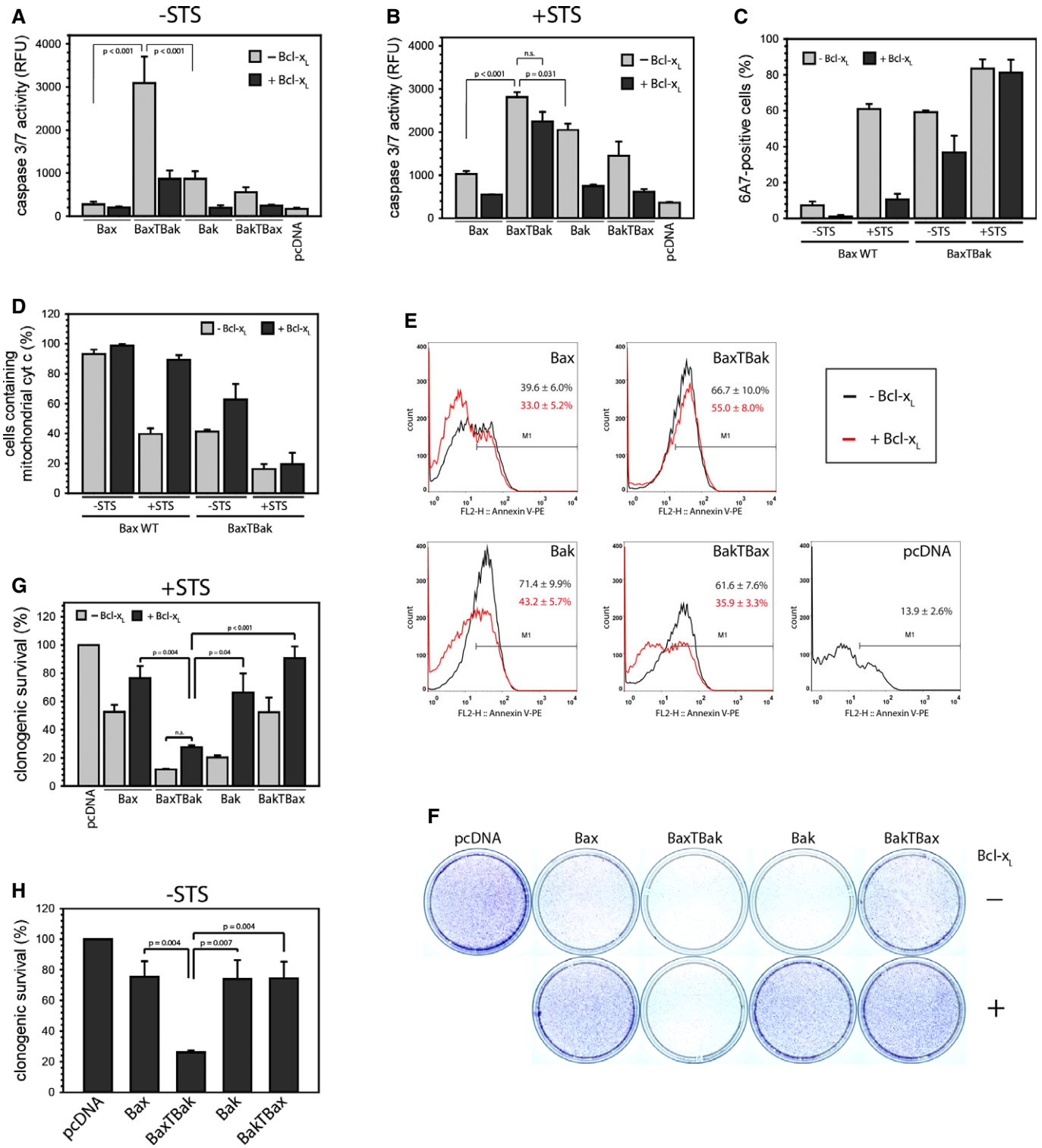

Figure 5.

rates. Bax is regulated by conformational changes (Hsu & Youle, 1997; Edlich *et al*, 2011). Therefore, the presence of the Bak MA might induce conformational changes and pre-activate Bax. We tested whether BaxTBak activity was likely based on pre-activation by conformational changes or increased MA hydrophobicity by comparing BaxTBak and its V197/198S variant (BaxTBakSS, Supplementary Fig S6A). Similar hydrophobicity of the MA of BaxTBakSS and Bax S184V suggests similar shuttling rates of both Bax variants, if MA hydrophobicity determines Bax/Bak retrotranslocation. Bax S184V is shifted to the mitochondria compared to wild-type Bax due to the exposure and the hydrophobicity of the MA (Nechushtan *et al*, 1999; Suzuki *et al*, 2000). In

Figure 5. Mitochondrial Bax is activated apoptosis stimulus-independently.

A Caspase-3/7 activity measured in HCT116 Bax/Bak DKO cells overexpressing Bax, BaxTBak, Bak or BakTBax with or without Bcl-x_L overexpression in the absence of apoptosis stimuli. Caspase activity is displayed in relative fluorescence units (RFU). pcDNA3.1-transfected cells served as a control. Data represent averages ± SEM. $n \geq 3$. *P*-values according to one-way ANOVA are displayed.

B Staurosporine (STS, 1 μM)-induced caspase-3/7 activity of Bax/Bak DKO cells overexpressing Bax, BaxTBak, Bak or BakTBax with or without Bcl-x_L overexpression displayed in relative fluorescence units (RFU). Data represent averages ± SEM. $n \geq 3$. *P*-values according to one-way ANOVA. BaxTBak activities with and without Bcl-x_L expression revealed no significant difference (n.s.), while in the presence of Bcl-x_L overexpression BaxTBak activity is significantly higher than Bax, Bak or BakTBax activities ($P < 0.001$).

C Analysis of the active Bax conformation in HCT116 Bax/Bak DKO cells expressing wild-type Bax or BaxTBak with (dark grey bars) or without Bcl-x_L overexpression (light grey bars) by the monoclonal antibody 6A7 (Sigma) detecting the active Bax protein fold by fluorescence imaging. Cells were analyzed prior to or after treatment with 1 μM STS in the presence of the pan-caspase inhibitor qVD. Data are represented as % of the expressing cell population ± SEM. $n = 4$.

D HCT116 Bax/Bak DKO cells ectopically expressing wild-type Bax or BaxTBak with (dark grey bars) or without Bcl-x_L (light grey bars) were analyzed in the presence or the absence of 1 μM STS and qVD for retained mitochondrial cyt *c*. Data are represented as % of the expressing cell population ± SEM. $n = 4$.

E Flow cytometry analysis of Annexin V staining of Bax/Bak DKO cells expressing Bax, BaxTBak, Bak or BakTBax in the absence (red line) or the presence of Bcl-x_L overexpression (black line), following STS treatment. The percentage of gated cells is displayed in the color of the corresponding graph. Data represent averages ± SD. $n = 4$.

F Colony formation of Bax/Bak DKO cells transfected with pcDNA, Bax, BaxTBak, Bak or BakTBax with or without Bcl-x_L overexpression. STS (1 μM) was added for 24 h before cells were replated and colonies were stained with methylene blue typically 14 days after treatment.

G Quantification of colony formation (F) of Bax/Bak DKO cells expressing Bax, BaxTBak, Bak or BakTBax with or without Bcl-x_L overexpression after STS treatment. Data represent averages ± SEM. $n = 4$. *P*-values according to one-way ANOVA. BaxTBak-expressing cells with or without Bcl-x_L expression showed no significant difference (n.s.).

H Quantification of the colony formation of Bax/Bak DKO cells expressing Bax, BaxTBak, Bak or BakTBax in presence of Bcl-x_L overexpression without apoptosis stimulation. Data represent averages ± SEM. $n = 5$. *P*-values according to one-way ANOVA.

fact, BaxTBakSS shuttles at similar rates as Bax S184V and significantly faster than BaxTBak, suggesting that Bax/Bak shuttling depends on the hydrophobicity but not the sequence of the MA (Fig 6A, Table 1). In contrast to BaxTBak, BaxTBakSS is not activated in the absence of apoptosis stimuli, suggesting BaxTBak activation based on MA hydrophobicity rather than pre-activation (Fig 6B–D).

The importance of the MA hydrophobicity for Bax/Bak shuttling demands an assessment of Bax and Bak with the same MA. To this end we made use of the Bax MA to avoid Bax activation at low shuttling rates. In addition, we introduced the S184V substitution to prevent MA binding to the hydrophobic Bax pocket, because substantial differences in the amino acid composition of Bak render similar interactions unlikely (Supplementary Fig S6A). The comparison of Bax, BakTBax and both variants containing the S184V substitution: (I) reveals the MA-independent influence of Bax and Bak on shuttling and commitment to apoptosis (Fig 6E), (II) indicates the effect of increased MA hydrophobicity, and (III) can indicate the contribution of MA binding to the hydrophobic pocket in Bax inhibition. The S184V substitution decreases the rate of Bax and BakTBax retrotranslocation (Supplementary Fig S6B; Table 1). The apoptotic activity of Bax S184V and BakTBax S184V is increased after apoptosis induction compared to the variants without S184V substitution (Fig 6F and G). Although both variants share only the C-terminal helix, Bax S184V and BakTBax S184V induce similar apoptosis signaling. Thus, the commitment of cells to apoptosis is determined by Bax/Bak shuttling rates. The retrotranslocation rates of both Bcl-2 proteins exhibit a linear dependence on the hydrophobicity, but not the sequence, of the MA (Fig 6H). Thus, the shuttling rates, localization and activity of Bax and Bak are determined by the hydrophobicity of the MA.

Membrane-integral Bcl-2 proteins are shuttled by retrotranslocation

Bak retrotranslocation, like Bax shuttling, is accelerated by the pro-survival Bcl-2 proteins, depending on the recognition of Bak BH3 by the pro-survival Bcl-2 protein. Bax shuttling involves the co-retrotranslocation of Bcl-x_L. Therefore we tested, whether the shuttling of Bax and Bak can shift the localization of Bcl-x_L accordingly. Indeed, wild-type Bcl-x_L shifts in the presence of Bax or Bak from the membrane-integral to the OMM-associated and cytosolic forms (Fig 7A; Supplementary Fig S7A). This Bcl-x_L migration from the OMM-integral form is not observed for the Bax/Bak shuttling-incompetent Bcl-x_LTBax (Fig 7B). This Bcl-x_L variant containing the Bax MA does not co-retrotranslocate Bax or Bak, but interacts with Bax in contrast to Bcl-x_L G138A (Fig 7B and C; Supplementary Fig S7B). Therefore, the Bcl-x_L localization is retrotranslocation-dependent. Although only a small fraction of wild-type Bax is present in the OMM-integrated protein pool of proliferating cells, larger pools of Bax variants (e.g. Bax S184V) are membrane integral and shuttle completely into the cytosol (Fig 7D; Supplementary Fig S7C and D; Edlich *et al*, 2011). Noteworthy, OMM-integral Bax is not in the active conformation (Supplementary Fig S7E).

These observations raise the question whether an increased mitochondrial Bax pool initiates apoptosis, although mitochondrial Bax accumulation is insufficient to commit cells to apoptosis (Valentijn *et al*, 2003; Todt *et al*, 2013). Perhaps extremely slow shuttling could raise levels above a required threshold. Thus, wild-type Bax expression was adapted to match the level of BaxTBak on the mitochondria (Fig 7E). Despite similar levels of wild-type Bax and BaxTBak on the OMM, in the absence of apoptotic stimuli, the release of the IMS protein Smac into the cytosol as well as PARP cleavage were only observed in cells expressing BaxTBak (Fig 7E and F). These results confirm that even high mitochondrial Bax levels do not per se commit cells to apoptosis. Bax activation seems to be determined by low rates of Bax shuttling, suggesting the importance of the dwell time of Bax molecules on the OMM. The co-retrotranslocation of Bcl-x_L and Bax or Bak involves the shuttling of the OMM-integral forms of Bax, Bak and Bcl-x_L into the cytosol (Fig 7G). Although Bax and Bak are shuttled by the same process from the OMM, cells must accelerate Bax retrotranslocation to prevent commitment to apoptosis at low Bax shuttling rates in the absence of apoptosis signaling.

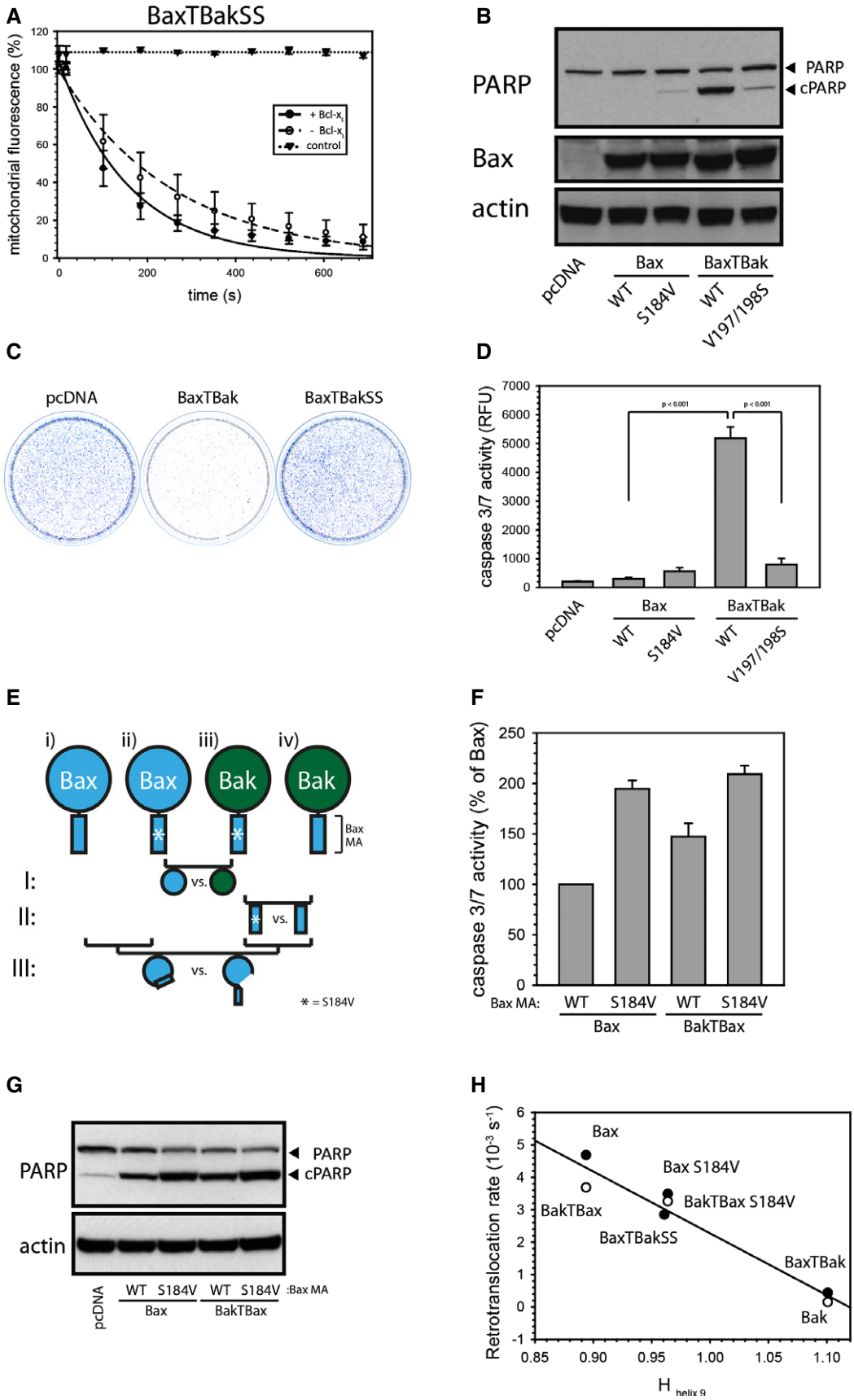

Figure 6.

Figure 6. Bax and Bak are regulated by the same mechanism.

A FLIP of BaxTBakSS without (○, broken line) or with (●, straight line) overexpressed Bcl-x_L. Fluorescence of a neighboring cell is shown as control (▼, dotted line). Data represent averages ± SEM from 20 (-Bcl-x_L) and 16 (+Bcl-x_L) ROI measurements.

B Western blot analysis of PARP cleavage in HCT116 Bax/Bak DKO cells overexpressing Bax, Bax S184V, BaxTBak or BaxTBak V197/198S. The experiment was carried out in the absence of apoptotic stimuli. Cells transfected with pcDNA3.1 vector serve as a control for PARP cleavage. Similar sample loading is controlled by actin. $n = 4$.

C Colony formation of Bax/Bak DKO cells transfected with pcDNA, BaxTBak or BaxTBakSS with Bcl-x_L overexpression in the absence of apoptotic stimuli. $n = 3$.

D Caspase-3/7 activity was measured in HCT116 Bax/Bak DKO cells overexpressing Bax, Bax S184V, BaxTBak or BaxTBak V197/198S. Caspase activity is displayed in relative fluorescence units (RFU). The experiment was carried out in the absence of apoptotic stimuli and pcDNA3.1-transfected cells served as a control. Data represent averages ± SEM. $n = 4$. *P*-values according to one-way ANOVA using the Holm–Sidak method are displayed.

E Comparison of Bax and Bak localization and activity dependent on the MA (Bax MA, blue rectangle) using wild-type Bax (i, blue), Bax S184V (ii), BakTBax (iv) and BakTBax S184V (iii). Direct comparison of Bax S184V (ii) and BakTBax S184V (iii) reveals MA-independent influences of Bax and Bak on protein localization and activity (I). The impact of the S184V substitution (*) on translocation, retrotranslocation and activity is shown by differences between BakTBax and BakTBax S184V (II). The additional effect of Bax MA binding to the hydrophobic groove of Bax (III) is indicated by the comparison of both Bax variants (i + ii) versus both Bak variants (iii + iv).

F STS-induced caspase-3/7 activity of HCT116 Bax/Bak DKO cells expressing Bax, Bax S184V, BakTBax or BakTBax S184V is displayed normalized to Bax activity. Data represent averages ± SEM. $n \geq 3$.

G Western blot analysis of PARP cleavage after STS treatment in HCT116 Bax/Bak DKO cells overexpressing Bax, Bax S184V, BakTBax or BakTBax S184V. Empty vector-transfected cells serve as a control for PARP cleavage and actin is the loading control. $n = 3$.

H Plot of Bax (●) and Bak (○) retrotranslocation rates versus hydrophobicity of the MA of the constructs. $R^2 = 0.95$.

Source data are available online for this figure.

Discussion

Bax and Bak share a common regulation of their localization and activity despite differences in their distribution between cytosol and mitochondria. Both pro-apoptotic Bcl-2 proteins are retrotranslocated by pro-survival Bcl-2 proteins from the mitochondria into the cytosol of healthy cells, dependent on the interaction with their BH3 motif. While Bax is retrotranslocated by Bcl-x_L, Bcl-2 and Mcl-1, Bcl-2 fails to accelerate Bak shuttling. This difference between Bax and Bak emphasizes the central role of retrotranslocation in Bax/Bak regulation, as Bcl-2 seems to be not involved in Bak regulation (Oltersdorf *et al*, 2005). The hydrophobicity of the C-terminal MAs of Bax or Bak determines different localization pattern and differential shuttling of both Bcl-2 proteins by the same retrotranslocation process (Figs 6H and 7G). Bax and Bak share similar apoptotic activity and subcellular localization at high shuttling rates, when the same MA is exposed, emphasizing the major role of the C-terminal MA in the regulation of Bax and Bak. Interestingly, retrotranslocation shuttles OMM-integral forms of Bax, Bak and Bcl-x_L that either are in equilibrium with OMM-associated protein or are directly shuttled into the cytosol by the retrotranslocation machinery. However, active Bax is not retrotranslocated, as Bax activation blocks shuttling into the cytosol (Edlich *et al*, 2011).

The analysis of Bax/Bak chimeras and variants revealed a potential requirement for increased Bax retrotranslocation in the absence of apoptosis signaling. Strikingly, the reduction of Bax retrotranslocation to the level of Bak shuttling initiates full Bax toxicity in the absence of apoptotic stimuli (Figs 5 and 7G). If the Bax shuttling rate is reduced sufficiently, mitochondrial Bax commits the cell to apoptosis in the absence of an apoptotic stress. Bcl-x_L overexpression does not prevent Bax activation at low shuttling rates. Therefore, the survival of the cell requires fast Bax retrotranslocation from the mitochondria. Bak, however, commits the cell to apoptosis only in the presence of apoptotic stimuli despite its predominant mitochondrial localization. Mitochondrial Bax activation adds to the differential regulation of both redundant proteins (Sarosiek *et al*, 2013). Bax activation probably only occurs with wild-type proteins when Bax shuttling is decreased, for instance, by BH3-only protein signaling (Edlich *et al*, 2011). However, the underlying mechanism of Bax activation at low shuttling rates remains to be solved. Mitochondrial Bax activation might explain cellular 'priming' to death by BH3-only proteins regardless of the presence of Bid, Bim and Puma (Ni Chonghaile *et al*, 2011; Vo *et al*, 2012). Depending on individual retrotranslocation rates, Bax activation could differ among different mitochondria, resulting in different organelle fates under stress conditions (Tait *et al*, 2010). On the other hand, mitochondrial Bax accumulation does not per se lead to Bax activation (Todt *et al*, 2013). Accordingly, similar levels of wild-type Bax and BaxTBak on the OMM result in BaxTBak but not Bax activity (Fig 7E). Under these conditions, both Bax variants most likely differ only in their translocation and retrotranslocation rates, and thus the residence time individual molecules spend on the mitochondria. Therefore, healthy cells inhibit Bax by accelerated shuttling into the cytosol and minimizing the time Bax molecules spend on the OMM in order to prevent Bax activation in the absence of apoptosis signaling.

Materials and Methods

Constructs

Bax and Bak constructs were cloned in pEGFP-C1 or pEYFP-C1 expression vectors. The chimeras BaxTBak and BakTBax were obtained by overlap-extension-PCR using the following primers: Bak–BaxTail rev (5′-CAAAGATGGTCACGGTGGGACCATTGCCC-3′); Bak–BaxTail for (5′-GGGCAATGGTCCCACCGTGACCATCTTTG-3′); XhoI–Bak for (5′-GCTACTCGAGCTATGGCTTCGGGGCAAG-3′); Bax–EcoRI rev (5′-GCGCGAATTCTCAGCCCATCTTCTTCCAGATG-3′); Bax–BakTail rev (5′-GCACGTTCAGGATCTGCCACGTGGGC-3′); Bax–BakTail for (5′-GCCCACGTGGCAGATCCTGAACGTGC-3′); XhoI–Bax for (5′-ATATCTCGAGCTATGGACGGGTCCGGGG-3′) and Bak–EcoRI rev (5′-GCGGGAATTCTCATGATTTGAAGAATCTTCGTACCACAAAC-3′). BaxTBak contains $Bax^{1–171}$ and $Bak^{188–211}$ and BakTBax consists of $Bak^{1–187}$ and $Bax^{172–192}$.

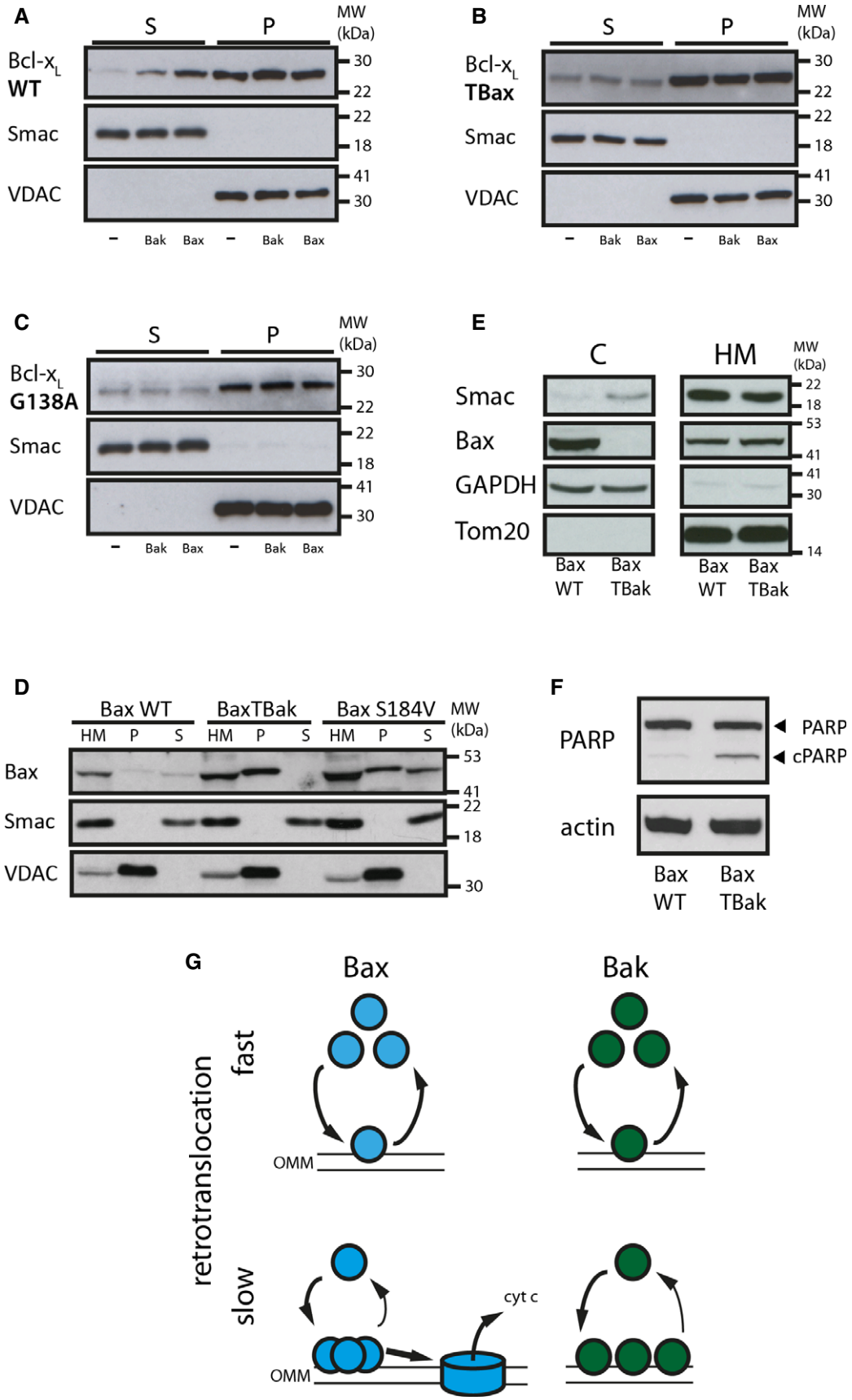

Figure 7.

Figure 7. Bax activation does not per se depend on mitochondrial Bax levels.

A Carbonate extraction of the HM protein fraction of Bax/Bak DKO cells expressing wild-type Bcl-x_L in the presence or the absence of Bak or Bax analyzed by Western blot. Carbonate-extractable supernatant (S) and membrane-integral protein pellet (P) are displayed. Smac is released from the IMS during carbonate extraction and VDAC remains in the pellet. $n = 4$.

B Western blot analysis of Bcl-x_LTBax carbonate extraction from the heavy membrane fraction from Bax/Bak DKO cells expressing the Bcl-x_L variant with or without Bak or Bax. Smac and VDAC serve as controls for supernatant (S) and pellet (P). $n = 4$.

C Carbonate extraction of the HM protein fraction of Bax/Bak DKO cells expressing Bcl-x_L G138A with or without Bak or Bax. Smac and VDAC serve as controls for supernatant (S) and pellet (P). $n = 4$.

D HM protein fraction and carbonate extraction of Bax/Bak DKO cells expressing Bax, BaxTBak or Bax S184V. Smac and VDAC serve as controls for supernatant (S) and pellet (P). $n = 4$.

E Smac release from the heavy membrane fraction (HM) into the cytosol (C) in HCT116 Bax/Bak DKO cells overexpressing Bax and BaxTBak without apoptotic stimuli analyzed by Western blot. Bax and BaxTBak were expressed differentially to produce similar levels of mitochondrial Bax. GAPDH and Tom20 serve as fractionation controls. $n = 3$.

F PARP cleavage in HCT116 Bax/Bak DKO cells expressing similar levels of mitochondrial Bax or BaxTBak analyzed by Western blot without apoptotic stimuli. Actin is used as a loading control. $n = 3$.

G Bax (blue, left) and Bak (green, right) are retrotranslocated similarly by pro-survival Bcl-2 proteins. The shuttling shifts also OMM-integral Bax and Bak into the cytosol dependent on the interactions between hydrophobic Bcl-x_L groove and BH3 motif of either Bax or Bak. While Mcl-1 shuttles Bax and Bak like Bcl-x_L, Bcl-2 only accelerates Bax retrotranslocation. Fast shuttling from the mitochondria into the cytosol at the level of Bax retrotranslocation in proliferating cells shifts Bax and Bak into the cytosol and inhibits their pro-apoptotic activities in parallel (top). Wild-type Bak is usually shuttled at low retrotranslocation rates and thus accumulates on the mitochondria (bottom, right). Shuttling at similar speed also causes mitochondrial Bax accumulation but results in stimulus-independent Bax activation (bottom, left). Therefore cells are required to shuttle Bax at high rates to prevent commitment to apoptosis.

Source data are available online for this figure.

Cell culture and transfection

HCT116 cells and HCT116 Bax/Bak DKO cells were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum and 10 mM Hepes in 5% CO_2 at 37°C. Cells were transfected with TurboFect (Fermentas) or Lipofectamine LTX (Invitrogen), typically with 100 ng of the wild-type or mutant constructs of Bax and Bak in pcDNA3.1 or pEGFP vector according to the manufacturer's instructions. In every comparative experiment the same amounts of all tested constructs were transfected in parallel using the same protocol resulting in similar transfection rates of approx. 90% (Supplementary Fig S2). Co-transfections with pcDNA3.1 Bcl-x_L were performed with a ratio of 1:6 between pro-apoptotic and pro-survival Bcl-2 protein. Cells were incubated for 6–8 h for confocal imaging.

LZRS-DD-Bcl-x_L was generated by inserting human Bcl-x_L into LZRS zeocin at EcoRI/XhoI sites and FKBP L106P in frame upstream via the EcoRI site using PCR/restriction digest based cloning (Banaszynski *et al*, 2006). HeLa cells expressing degradation-prone DD-Bcl-x_L were generated by retroviral transduction as follows. Amphotropic Phoenix cells (0.5×10^6 in a 10-cm dish) were transfected with LZRS DD Bcl-x_L using Lipofectamine 2000 (Invitrogen). Two days later virus-containing supernatant was harvested, filtered and used to infect target HeLa cells in the presence of polybrene (1 μg/ml). Two days post-infection, stably expressing cells were selected by growth in zeocin (200 μg/ml, Invitrogen).

Confocal microscopy and FLIP

HCT116 Bax/Bak DKO cells were seeded on a chambered cover glass (Thermo Scientific) in McCoy's 5A medium, grown for 36 h and transfected for 6–8 h. Cells were then incubated with MitoTracker far red for 10 min and imaged using a Zeiss 510 META confocal LSM microscope equipped with argon (458/488/514 nm lines) and HeNe (543/633 nm) lasers.

Fluorescence loss in photobleaching (FLIP) experiments were performed as described previously (Edlich *et al*, 2011). In short, cells were imaged prior to bleaching. Then a single region (diameter of 1 μm) within the nucleus was repeatedly bleached with two iterations of a 488 nm laser line (100% output) using a Zeiss LSM 510 META with a 63× PlanFluor lens. Two images were collected after each bleach pulse, with 30 s between pulses. After 15 cycles of bleaching and collecting 30 images, separate measurements on the mitochondria were taken to analyze loss in fluorescence. Unbleached cells neighboring analyzed cells served as controls for photobleaching during image acquisition of each measurement. Fluorescence intensities were normalized by setting the pre-bleach fluorescence to 100% signal.

Apoptosis activity assays

For caspase-3/7 measurements, HCT116 Bax/Bak DKO cells were seeded in 6-well plates and transfected with the same amount of plasmids containing different Bax or Bak variants in the presence or absence of Bcl-x_L. Then, cells were treated with 1 μM STS or left untreated. After 12 h incubation, whole-cell lysates were isolated and fluorogenic caspase-3 substrate N-acetyl-DEVD-AMC (BD Pharmigen) was added to each sample according to manufacturer's protocol. Samples were incubated for 1 h at 37°C and then fluorescence signal was measured 30 times at 2-min intervals with an excitation wavelength of 355 nm and an emission wavelength of 460 nm in a plate reader. The same samples were also subjected to analysis of PARP cleavage by Western blot.

Whole-cell lysis and subcellular fractionation

Cells were harvested and incubated in cell lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0,5% Triton X-100, protease inhibitor cocktail) for 15 min on ice. Whole-cell extracts were obtained by centrifugation at 15,000 × *g* for 10 min at 4°C. Protein concentrations were determined using a Bradford Assay (RotiQuant, Roth). Samples were boiled in SDS sample buffer for 10 min at 95°C and subsequently subjected to SDS–PAGE and Western Blot analysis. Subcellular fractionations were performed as previously described (Todt *et al*, 2013).

Carbonate extraction

Mitochondrial pellets were prepared as described above and resuspended in 100 mM Na_2CO_3 at pH 11.5. The samples were incubated on ice for 20 min to disrupt protein–protein interactions of peripheral proteins while interactions protected by the lipid bilayer like lipid–protein interactions remain intact. Then, the membranes were pelleted at 150,000 × *g* for 30 min at 4°C. The supernatant, containing membrane-associated proteins, was subjected to protein precipitation by acetone. The pellet was resuspended once more in 100 mM Na_2CO_3 at pH 11.5 and incubated on ice for 20 min. Finally, the resuspended samples were centrifuged at 155,000 × *g* for 30 min at 4°C to obtain carbonate inextractable proteins. Both fractions were assayed by Western blot.

Clonogenic survival assay

For the evaluation of clonogenic survival, HCT116 Bax/Bak DKO cells were seeded in 6-well plates. After the transfection with different Bax or Bak variants in the presence or the absence of Bcl-x_L, 1 μM STS was applied to each sample for 24 h or cells were left untreated. After 12–16 days, surviving colonies were fixed and stained with 1% methylene blue.

FACS analysis

Cells were seeded in 6-well plates and transfected with equal DNA amounts of Bax or Bak and pcDNA or Bcl-x_L. Cells were harvested and washed with PBS. Cells were incubated in Annexin V binding buffer substituted with Annexin V–PE (eBioscience) for 10 min at room temperature. Finally, cells were fixed with 1% paraformaldehyde (neoLab) and measured using a BD FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo Version 10.0.7.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

FT, ZC and FR designed and performed experiments, analyzed data and wrote the paper. FEm, JL, GI and AK performed experiments and analyzed data. SWGT performed experiments, analyzed data and edited the paper. SF and HFL analyzed data and helped to conceptualize the project. FEd conceptualized the project, designed experiments, analyzed data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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